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Formation of Cellulose by Certain Species of *Acetobacter*

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Brown (1886) showed that the surface membranes (often designated pellicles) formed by *Bacterium xylinum* when grown on suitable carbohydrate media consist of cellulose, and this material has been examined by Hibbert & Barsha (1931), Tarr & Hibbert (1934), Khouvine, Champetier & Sutra (1932), Sisson (1936), Franz & Schiebold (1943), Frey-Wyssling & Mühlethaler (1946) and by Hestrin, Ashner & Mager (1947). *Bact. xylinum* is now known as *Acetobacter xylinum*. Frey-Wyssling & Mühlethaler (1946) have shown also that the pellicle formed by *Acetobacter xylinoides* Henneberg from glucose consists of cellulose, but the surface covers which are formed from certain substrates by *A. pasteurianum* Hansen, *A. kützingianum* Hansen and *A. acetigenum* Henneberg, have not hitherto been subjected to detailed examination.

In this laboratory, while studying bacteria from an East African vinegar brewery, Dr D. Kulka isolated a strain of *A. acetigenum* (hereafter referred to as *A. acetigenum* E.A.) which produced very thick pellicles on glucose media. Chemical examination showed the pellicle material consisted of cellulose and this was confirmed by X-ray examination. The results of the X-ray examination are reserved for a future communication.

The production of cellulose by this organism, by *A. acetigenum* (type culture), by *A. pasteurianum* and by *A. kützingianum*, is now described.

EXPERIMENTAL AND RESULTS

Cultural conditions governing the formation of cellulose

Pellicles which gave reactions for cellulose were formed by *A. acetigenum* E.A. from soluble starch, dextrin, sucrose, maltose, lactose, glucose, fructose, galactose, α-methyl-D-glucoside, β-methyl-D-glucoside, salicin, arabinose, xylose, rhamnose, mannitol, erythritol, glycerol, and ethylene glycol, respectively. Tarr & Hibbert (1931) reported the inability of *A. xylinum* to form cellulose from the α- and β-methyl-D-glucosides, three pentoses, erythritol and ethylene glycol, respectively. They did not ascertain whether their organism could utilize salicin.

A. acetigenum E.A., *A. acetigenum* (Henneberg) Bergey *et al.*, National Collection of Type Cultures (N.C.T.C.) 5346, *A. pasteurianum* (an atypical strain) N.C.T.C. 613 and *A. kützingianum* (Hansen) Bergey *et al.*, N.C.T.C. 3924, produced cellulose in a medium (Henneberg, 1926) consisting of $(NH_4)_2SO_4$, 3 g.; KH_2PO_4, 3 g.; $MgSO_4.7H_2O$, 2 g.; glucose, 20 g. and ethanol (added after sterilization) 25 g., together with water to 1 l. Equally vigorous or more profuse growth could be obtained by substituting corn-steep liquor (C.S.L.), 1–2% (v/v), or Witte's peptone 0·2% (w/v) in place of or in addition to the ethanol. The same adjuncts also stimulated growth in yeast-water (made by boiling 75 g. of brewery pressed yeast for 30 min. in 1 l. of water) to which glucose or another suitable carbohydrate had been added. In some cases addition of sterile $CaCO_3$ (precipitated chalk) improved the yield of cellulose. In media containing

ethylene glycol, however, the addition of $CaCO_3$ prevented cellulose formation.

The cultures were developed in the flat, circular, glass dishes formerly employed in penicillin production (Clayton, Hunwicke, Hanes, Robinson & Andrews, 1944); these were charged with medium to a depth of 1 cm. and, after inoculation, were incubated at 30°.

When *A. acetigenum* E.A. was grown on glucose in yeast-water the yield of cellulose was improved by addition both of $CaCO_3$ and C.S.L. (Table 1). The yields from some substances other than glucose are given in Table 2. Yeast-water was employed in two only of the cases shown in Table 2; in the other cases the volume was made up to bulk with tap water. In all cases the dishes stood without agitation during incubation.

Table 1. *Yields of cellulose formed by* Acetobacter acetigenum *E.A. from glucose*

Composition of medium (to 200 ml. with yeast-water)	Time of incubation at 30° (days)	Purified cellulose: Yield (g.)	Purified cellulose: As % of wt. of glucose initially present
Glucose, 5 g.	10	0·11	2·2
Glucose, 5 g.; $CaCO_3$, 2 g.	10	0·42	8·4
Glucose, 10 g.	12	0·18	1·8
Glucose, 10 g.; $CaCO_3$, 2 g.	12	0·50	5·0
Glucose,,10 g.; $CaCO_3$, 2 g.; C.S.L. 4 ml.	10	0·71	7·1
Glucose, 10 g.; C.S.L., 4 ml.	15	0·64	6·4
Glucose, 36 g.; $CaCO_3$, 2 g.	10	1·08	3·0

Chemical examination of the bacterial cellulose

Both strains of *A. acetigenum* gave higher yields of pellicle material than were obtained from corresponding cultures of *A. pasteurianum* and *A. kützingianum*. All the pellicles formed from the different substrates by *A. acetigenum* E.A. and those formed from glucose by *A. acetigenum* N.C.T.C. 5346, by *A. pasteurianum* N.C.T.C. 613 and by *A. kützingianum* N.C.T.C. 3924, after purification, gave the following reactions for cellulose: they were soluble in H_2SO_4 (72%, w/w) and in cuprammonium sulphate, being precipitated in flocculent condition on addition of excess of acidulated water. They were soluble also in a solution of $ZnCl_2$ in HCl and they could be bleached like cotton and dyed with direct cotton dyes such as congo red, benzopurpurin and sky-blue F.F.S. They could be hydrolysed by H_2SO_4 with production of a substance giving reactions of glucose.

Membranes from cultures of *A. acetigenum* E.A., *A. pasteurianum* and *A. kützingianum* on glucose media were purified by thorough washing with distilled water, treatment for 10 hr. with cold aqueous NaOH solution (2%, w/v), followed by further washings with water, dilute acetic acid and, finally, with distilled water for 12 hr. They were then dried in air and finally in a vacuum desiccator.

(Material formed by *A. acetigenum* E.A.: Found: C, 44·7; H, 6·7; by *A. pasteurianum*: Found: C, 45·3; H, 6·8; by *A. kützingianum*: Found: C, 43·8; H, 6·4; calc. for $(C_6H_{10}O_5)_n$: C, 44·5; H, 6·2%.)

Pellicle material from cultures of *A. acetigenum* E.A. on glucose and glycerol media, respectively, was purified in the manner stated above and was treated as follows:

(*a*) *Conversion to glucose.* Membrane (0·5 g.) from a glucose culture was dissolved in H_2SO_4 (72%, w/w) and the solution was diluted with fifty times its volume of water and boiled for 5 hr. with periodical addition of sufficient water to maintain the original volume. After neutralization with Na_2CO_3, samples reduced Fehling's solution, Barfoed's reagent and ammoniacal $AgNO_3$ solution. The bulk of the boiled liquid was filtered and divided into two portions, one of which, on appropriate treatment with phenylhydrazine acetate, yielded phenylglucosazone, m.p. 209° (decomp.), identical in crystalline form with an authentic specimen prepared from pure glucose. The other portion was warmed for several hours with 2:4-dinitrophenylhydrazine in 2N-HCl and the orange-red, solid product after recrystallization had m.p. 264° (decomp.) and was indistinguishable from the 2:4-dinitrophenylosazone of glucose (m.p. 264°, decomp.) prepared from British Drug Houses Ltd. glucose (A.R.). (Found: N, 20·6. Calc. for $C_{18}H_{18}O_{12}N_8$: N, 20·8%.)

The membrane from a culture on a glycerol medium was purified and dried in a vacuum desiccator. (Found: C, 44·2; H, 6·6. Calc. for $(C_6H_{10}O_5)_n$: C, 44·5; H, 6·2%.) A portion

Table 2. *Yields of cellulose formed by* Acetobacter acetigenum *E.A. from substances other than glucose*

	Composition of medium (to 200 ml.)	Days of incubation at 30°	Purified cellulose: Yield (g.)	Purified cellulose: As % of wt. of carbon source initially present
1	Mannitol, 10 g.; Henneberg salts; C.S.L., 4 ml.	28	0·47	4·7
2	Glycerol, 10 g.; Henneberg salts; C.S.L., 4 ml.	28	0·35	3·5
3	Glycerol, 20 g.; Henneberg salts; C.S.L., 4 ml.	35	1·00	5·0
4	Ethylene glycol, 10 g.; Henneberg salts; C.S.L., 4 ml.	28	0·10	1·0
5	Ethylene glycol, 15 g.; C.S.L., 4 ml.; yeast-water	35	0·90	6·0
6	Arabinose, 5 g.; Henneberg salts (with the weight of $(NH_4)_2SO_4$ reduced to 1 g./l.); peptone, 0·4 g.	23	0·12	2·3
7	Xylose, 5 g. Other constituents as for arabinose	23	0·11	2·2
8	Rhamnose, 5 g. Other constituents as for arabinose	23	0·10	2·0
9	Erythritol, 5 g.; C.S.L., 4 ml.; yeast-water	23	0·10	2·0
10	Soluble starch, 3 g.; K_2HPO_4, 0·6 g.; peptone, 0·4 g.	34	0·3	10·0

of it was hydrolysed with H_2SO_4, yielding a solution from which the phenylosazone (m.p. 209°) and the 2:4-dinitrophenylosazone of glucose (m.p. 264°) were prepared.

(*b*) *Conversion to a mixture of octa-acetyl cellobiose and α-penta-acetyl glucose.* Membrane (1 g.) from a culture on a glucose medium was treated with acetic anhydride (8 ml.) and conc. H_2SO_4 (0·2 ml.) during 16 days at 45°. The product was isolated by the procedure of Barsha & Hibbert (1934) and purified. Yield, 0·7 g. of m.p. 226°. The mixed melting point with an authentic sample of octa-acetyl cellobiose (m.p. 226°), prepared from purified cotton wool, was identical. In a further experiment 2 g. of membrane were acetolysed with acetic anhydride (16 ml.), glacial acetic acid (8 ml.) and conc. H_2SO_4 (2 ml.) at room temperature for 24 days. The crude product (dry wt. 1·2 g.) yielded 0·3 g. of pure octa-acetyl cellobiose (m.p. 226°) and 0·2 g. of α-penta-acetyl glucose of m.p. 110°. The mixed melting point of the latter with an authentic sample of α-penta-acetyl glucose kindly supplied by Prof. F. Challenger was identical.

(*c*) *Conversion to cellulose triacetate.* The material (1·1 g.) obtained from a glucose medium was boiled gently for 2 hr. with acetic anhydride (20 ml.), glacial acetic acid (10 ml.) and fused $ZnCl_2$ (1 g.). On pouring the mixture into water the product separated, and was purified by addition of methanol to its solution in acetone, giving 1·6 g. (96% yield) of cellulose triacetate which was indistinguishable from the product prepared by the same method from 1·2 g. of purified cotton wool. Portions of each product were subjected to further purification by precipitation from solution in $CHCl_3$ by addition of methanol. (Acetate from bacterial cellulose: Found: C, 49·1; H, 5·8; $CH_3.CO$–, 44·7; acetate from cotton wool: Found: C, 49·2; H, 5·5; $CH_3.CO$–, 45·5; Calc. for $C_6H_7O_5(-CO.CH_3)_3$: C, 50·0; H, 5·6; $CH_3.CO$–, 44·8%.)

(*d*) *Acetolysis at low temperature with formation of β-penta-acetyl glucose.* Acetic anhydride (8 g.), glacial acetic acid (4 g.) and conc. H_2SO_4 (1 ml.) were mixed at −10° and 1 g. of membrane from a culture on a glycerol medium was added in small portions, the temperature not being allowed to exceed 0°. After several hours at 0° the material had dissolved and 16 days later the solution was poured into water (400 ml.). The weight of the white flocculent precipitate after washing and drying was 0·5 g. It was dissolved in $CHCl_3$, 3 vol. of methanol were added, and the whole was filtered and concentrated, yielding finally a small quantity of crystalline material. This was redissolved in $CHCl_3$, boiled with charcoal and allowed to stand at room temperature, when crystals of m.p. 129–133° were obtained. The mixed melting point with an authentic sample of β-penta-acetyl glucose, kindly supplied by Prof. F. Challenger, was identical.

Detection of intermediates during the formation of cellulose

Identification of dihydroxyacetone (or glyceraldehyde) in cultures on a glycerol medium. In a medium consisting of glycerol (10%, w/v) in Henneberg's solution of salts together with C.S.L. (2%, v/v) *A. acetigenum* E.A. formed a substance which reduced Fehling's solution in the cold. The reducing value of the culture liquid, estimated by the Willstätter-Schudel method, showed a considerable rise on the 35th day. At this time accumulation of cellulose had ceased. In another experiment, material in solution which could be precipitated by 2:4-dinitrophenylhydrazine was so removed periodically, and at each of these times the weight of cellulose was ascertained (Table 3). All the precipitates after crystallization from pyridine were obtained as small red prisms of m.p. 284°. The mixed melting point with a sample of the 2:4-dinitrophenylosazone of dihydroxyacetone (or of glyceraldehyde), prepared from Merck's dihydroxyacetone, was identical. (Found: N, 25·3. Calc. for $C_{15}H_{12}O_8N_8$: N, 25·0%.) From similar glycerol cultures the triose was obtained as the phenylosazone, which crystallized from benzene in golden-yellow needles of m.p. 132°. The mixed melting point with an authentic specimen (m.p. 132°) prepared from Merck's dihydroxyacetone was identical. (Found: C, 66·8; H, 6·2; N, 21·0. Calc. for $C_{15}H_{16}ON_4$: C, 67·2; H, 6·0; N, 20·9%.)

Table 3. *Production of cellulose and of dihydroxyacetone by* Acetobacter acetigenum *E.A. in 200 ml. of a medium containing glycerol (10%, w/v)*

Time of incubation at 30° (days)	Purified cellulose: Yield (g.)	Purified cellulose: As % of wt. of glycerol initially present	Wt. of 2:4-dinitrophenylosazone of dihydroxyacetone (g.)
17	0·70	3·5	1·68
20	0·82	4·1	1·72
28	0·83	4·2	1·78
35	0·97	4·9	1·84
49	0·90	4·5	3·60

Identification of glycolaldehyde in cultures on an ethylene glycol medium. Cultures were developed in media containing (*a*) ethylene glycol, 21 g.; $CaCO_3$, 9 g.; C.S.L., 4 ml.; yeast-water to 600 ml.; (*b*) ethylene glycol, 21 g.; $CaCO_3$, 9 g.; yeast-water to 600 ml.; and (*c*) ethylene glycol, 14 g.; C.S.L., 4 ml. yeast-water to 400 ml.

After 15 days at 30° a thick pellicle had developed on (*c*), but (*a*) and (*b*) were without surface growth. The pellicle from (*c*) after washing and drying weighed 0·92 g., equivalent to 6·6% on the glycol initially in solution. Each medium was filtered and in all cases samples on treatment with HCl and naphthoresorcinol gave the colour reaction for glyoxylic acid (Neuberg, 1910). Each filtrate was then mixed with twice its volume of a solution of 2:4-dinitrophenylhydrazine in 2N-HCl; the resultant precipitates weighed (*a*) 0·2042 g., (*b*) 0·0520 g. and (*c*) 0·2878 g., respectively. Precipitates (*a*) and (*c*) were recrystallized first from pyridine, then from nitrobenzene, after which each had m.p. 326° (decomp.). In each case the mixed melting point with an authentic sample of the 2:4-dinitrophenylosazone of glycolaldehyde (m.p. 326–329°, decomp.) prepared from glycolaldehyde obtained by the method of Fenton & Jackson (1899) was 326° (decomp.). (Found: N, 26·4, 26·6. Calc. for $C_{14}H_{10}O_8N_8$: N, 26·8%.)

In another experiment glycolaldehyde was isolated from an ethylene glycol medium as the phenylosazone which, after crystallization from absolute ethanol, had m.p. 170° (decomp.). The mixed melting point with a sample prepared from authentic glycolaldehyde (Fenton & Jackson, 1899) was identical. (Found: C, 70·4; H, 5·9. Calc. for $C_{14}H_{14}N_4$: C, 70·6; H, 5·8%.)

DISCUSSION

The formation of cellulose from ethylene glycol by way of glycolaldehyde, as now described, is of interest principally from the fact that, so far as the authors are aware, it constitutes the first instance in which the transformation of this aldehyde to a higher carbohydrate has been established experimentally by use of living cells. The only previously recorded observation of the conversion of ethylene glycol to glycolaldehyde by a micro-organism appears to be that of Goepfert & Nord (1942) who isolated glycolaldehyde, in the form of its dimedone derivative, from a culture of a *Fusarium* species on a glycol medium. The bacterial conversion of glycerol, via dihydroxyacetone (or glyceraldehyde) to cellulose, is likewise of interest, since glyceraldehyde has not hitherto been identified as a metabolite in reactions leading to cellulose.

SUMMARY

1. A strain of *Acetobacter acetigenum*, isolated from East African vinegar, formed cellulose from all of eighteen carbohydrates, or alcohols related to carbohydrates, which were submitted to its action.

2. By its ability to convert to cellulose the α- and β-methyl-D-glucosides, three pentoses, erythritol and ethylene glycol, respectively, the new organism showed itself to be enzymically more active than *Acetobacter xylinum* which, in previous studies of cellulose formation (Tarr & Hibbert, 1931), proved unable to utilize these substances for this purpose.

3. During the formation of cellulose from ethylene glycol and from glycerol there were formed glycolaldehyde and glyceraldehyde, respectively, and these were characterized as their osazones.

4. It was shown that *A. acetigenum* Henneberg, *A. pasteurianum* (an atypical strain) and *A. kützingianum* Hansen can each synthesize cellulose when grown on suitable media.

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Studies in Detoxication

37. METABOLISM OF BENZENE. EXAMINATION OF THE GLUCURONIDE FRACTION OF RABBIT URINE AFTER ADMINISTRATION OF BENZENE. ISOLATION OF PHENYLGLUCURONIDE

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In 1881 Schmiedeberg obtained a gum from the urine of dogs fed with benzene and concluded that because the gum gave phenol and tests for glucuronic acid, it contained phenylglucuronide, a compound which had not then been described and which was isolated for the first time by Külz in 1890 from the urine of rabbits fed with phenol.

No glucuronide has, hitherto, been isolated from urine after feeding benzene. The urine passed after feeding benzene will be referred to in this paper as benzene urine. Porteous & Williams (1949*b*) examined the ethereal sulphate fraction of benzene urine and found it to contain phenol, catechol, quinol and hydroxyquinol. We have now examined the